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Stable expression of shRNAs in human CD34$^+$ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*

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RNA interference occurs when cytoplasmic small interfering RNAs (siRNAs) enter the RNA-induced silencing complex and one strand guides cleavage of the target RNA by the Argonaute 2 protein[1–3]. A significant concern when applying siRNAs or expressing small hairpin RNAs (shRNAs) in human cells is activation of the interferon (IFN) response[4–10]. Synthetic siRNAs harboring certain motifs can induce an immune response when delivered to mouse and human immune cells such as peripheral blood mononuclear cells, monocytes, plasmacytoid dendritic cells (pDCs) and nonplasmacytoid dendritic cells (mDCs)[11–13]. In the present study we have tested the immunostimulatory effects of lipid-delivered siRNAs versus Pol III promoter–expressed shRNAs in primary CD34$^+$ progenitor–derived hematopoietic cells. We show that in this system, lipid-delivered siRNAs are potent inducers of IFNα and type I IFN gene expression, whereas the same sequences when expressed endogenously are nonimmunostimulatory.

The observation of siRNA-induced immune responses in immune cells has raised concerns about the possible side effects of RNA interference, such as the potential for activation of inflammatory cytokine production (tumor necrosis factor (TNF)α, interleukin (IL)-6 and IL-12) and the IFN response. Transfection of human monocytes with synthetic siRNA induces TNFα and IL-6 (ref.13), and transfection of peripheral blood mononuclear cells with siRNA and shRNA induces IFNα, IL6 and TNFα[11–13]. The induction of immune responses to RNA occurs in mice and humans via immune-cell subtypes and depends on sequence and cell type[14]. Certain sequence motifs (5′-GUCCUUCAA-3′ and 5′-UGUGU-3′) in synthetic siRNA/shRNA and even in the ssRNAs that constitute the siRNAs induce cytokine production, particularly IFN in pDCs via Toll-like receptor 7 (TLR7)[12] and TNFα in monocytes, probably via TLR8 (refs. 11,15). It has been shown that a GU-rich RNA is immunostimulatory in both murine and human immune cells and activates NF-κB when delivered in a lipid vehicle at high concentrations to TLR8-expressing HEK293 cells, suggesting that RNAs containing GU sequences are more likely to be immunostimulatory[15]. The recognition of RNA danger signals via TLR7 and TLR8 seems to depend on compartmentalization of the RNA in the endosome and not on self versus nonself signals[11–13,15].

A potential therapeutic application of RNA interference is treatment of HIV-1 in a gene therapy setting. One approach for carrying this out involves the introduction of anti-HIV–encoding shRNAs into CD34$^+$ hematopoietic stem cell progenitors for transplantation into human subjects. Because transfection with siRNAs and shRNAs can induce the production of inflammatory cytokines by human immune cells[11–13], we hypothesized that a similar immune response to these RNAs might be produced in human immune cells derived from CD34$^+$ progenitor cells.

We cultured CD34$^+$ progenitor cells in the presence of macrophage colony stimulating factor (M-CSF) and granulocyte macrophage (GM)-CSF to induce differentiation to a mixed population of immune cells containing monocytes, mDCs and pDCs. After 7 d of stimulation, the cell population contained ~35% CD14$^+$ monocytes, 8% CD11c$^+$/BDCA1$^+$ myeloid dendritic cells and 13% CD11c-/BDCA4$^+$ pDCs (**Supplementary Fig. 1a** online). To determine whether IFN-inducible gene expression could be activated *in vitro* in CD34$^+$ progenitor–derived immune cells, we added IFNα in varying concentrations (100 U/ml to 1,000 U/ml) to the culture medium and measured IFNα-responsive expression of *IFNβ*, *OAS1*, *MX1* (also known as *MxA*), *G1P2* (also known as *ISG15*) and *CDKL2* (also known as *P56*) mRNAs by real-time PCR. As little as 100 U/ml of exogenous IFNα stimulated a strong upregulation of the IFN-inducible genes *OAS1*, *MX1*, *G1P2* and *CDKL2* over levels in control cells, but no appreciable increase in *IFNβ* mRNA expression was observed (**Fig. 1a**).

To examine the response of CD34$^+$ progenitor–derived immune cells to dsRNA, we transfected the cells with polyinosinic:polycytidylic acid (polyIC) or two different siRNAs and performed IFNα enzyme-linked immunosorbent assay (ELISA) on supernatants 48 h after transduction (**Figs. 1** and **2**). Real-time PCR was performed for *IFNβ* mRNA and the IFN-inducible mRNAs *OAS1*, *MX1*, *G1P2* and *CDKL2*. The cells express *IFNβ* mRNA when transfected with polyIC in addition to the IFN-inducible *OAS1*, *MX1*, *G1P2* and *CDKL2*

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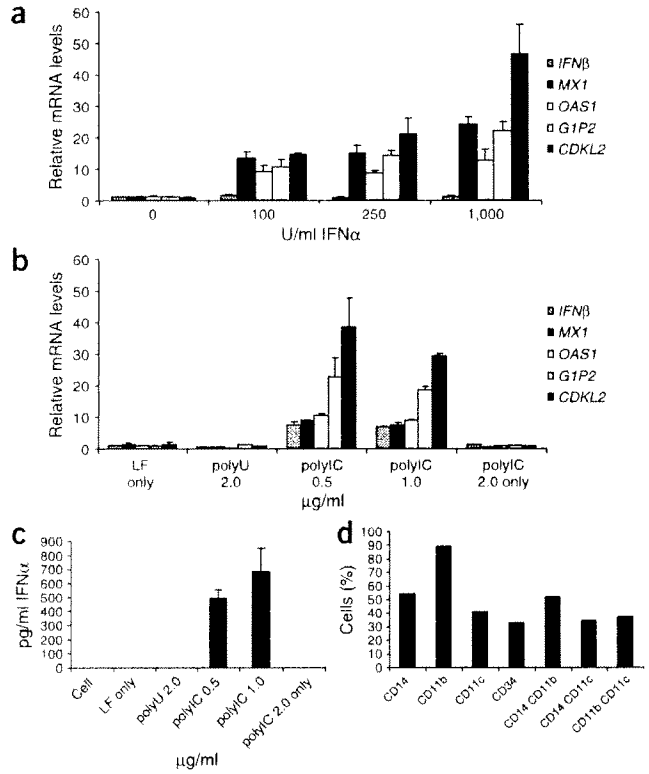

Figure 1 CD34$^+$ progenitor-derived immune cells produce IFN and IFN-inducible gene expression in response to treatment with IFN or to transfection with polyIC. (**a**,**b**) Real-time PCR performed for *MX1*, *OAS1*, *G1P2*, *CDKL2* and *IFNβ* mRNAs extracted from CD34$^+$ progenitor cells isolated from cord blood. Cells were cultured with M-CSF and GM-CSF for 3 d and then 100–1,000 U/ml IFNα was added to the medium (**a**) or for 6 d at which time 2 μg/ml of polyIC only, or 0.5 or 1.0 μg/ml polyIC in complex with Lipofectamine 2000 was added to the cells (**b**). Total RNA was extracted 2 d later and expression of HPRT1 mRNA was used for normalization. (**c**) IFNα ELISA was performed on supernatants of cells from **b**. One representative experiment with triplicates is shown. Error bars represent s.d. Results are from two experiments. (**d**) FACS analysis for CD14, CD11b, CD11c and CD34 was performed on CD34$^+$ progenitor-derived monocytes 6 d after addition of M-CSF and GMCSF for the experiment in **b**.

mRNAs (**Fig. 1b,c**). In IFNα-treated cells and cells transfected with polyIC, CDKL2 was the most strongly upregulated IFN-inducible gene product. IFNα protein (700-1200 U/ml) was produced in CD34 progenitor–derived immune cells transfected with polyIC (**Fig. 1c**). No IFNα protein or IFN-inducible gene expression was observed in cells treated with the lipid vehicle alone or polyIC alone, whereas transfection with up to 10 μg/ml polyU RNA induced only low amounts of IFNα (**Fig. 1b,c**). Fluorescence-activated cell sorting (FACS) analysis of the CD34$^+$ progenitor–derived immune cells showed that the cells differentiated into a mixed population of monocytes and dendritic cells (**Figs. 1d** and **2d**).

To determine whether and to what extent the siRNAs to site I (tat/rev) and site II (rev) were immunostimulatory, we transfected CD34$^+$ progenitor–derived immune cells with 35 nM (0.5 μg/ml) of synthetic siRNA to either site I or site II. Strong upregulation of the IFN-inducible genes *OAS1*, *MX1*, *G1P2* and *CDKL2* was observed for both siRNAs (**Fig. 2a**). *IFNβ* mRNA expression was not increased to the same level by transfection with siRNA as transfection with polyIC. IFNα ELISA indicated that synthetic siRNAs to site I and site II caused production of IFNα protein (600–1,000 pg/ml) 48 h after transfection of CD34$^+$ progenitor–derived monocytes (**Fig. 2b**). The levels of IFNα induction by synthetic siRNA to site II were similar to those induced by polyIC. siRNA to site II induced a 40% stronger induction of IFNα than siRNA to site I, consistent with its longer UGUG motif. Lipid vehicle alone did not induce IFNα protein or the upregulation of IFN-inducible gene expression (**Fig. 2a,b**).

We examined CD34$^+$ progenitor cell–derived immune cells expressing shRNA to site I (tat/rev) or site II (rev), and a triple construct (containing site I shRNA, a chimeric VA1-CCR5 ribozyme and a nucleolar-localizing TAR decoy) (**Supplementary Fig. 2** online) for induction of *IFNβ* mRNA and IFN-inducible expression of *OAS1*, *MX1*, *G1P2* and *CDKL2* at 3 weeks after transduction. Expression of the triple construct, containing the shRNA for site I, induced no measurable increase in *OAS1*, *MX1*, *CDKL2*, *G1P2* or *IFNβ* mRNA over levels in control cells (**Fig. 3a**). Expression of the shRNAs to site I

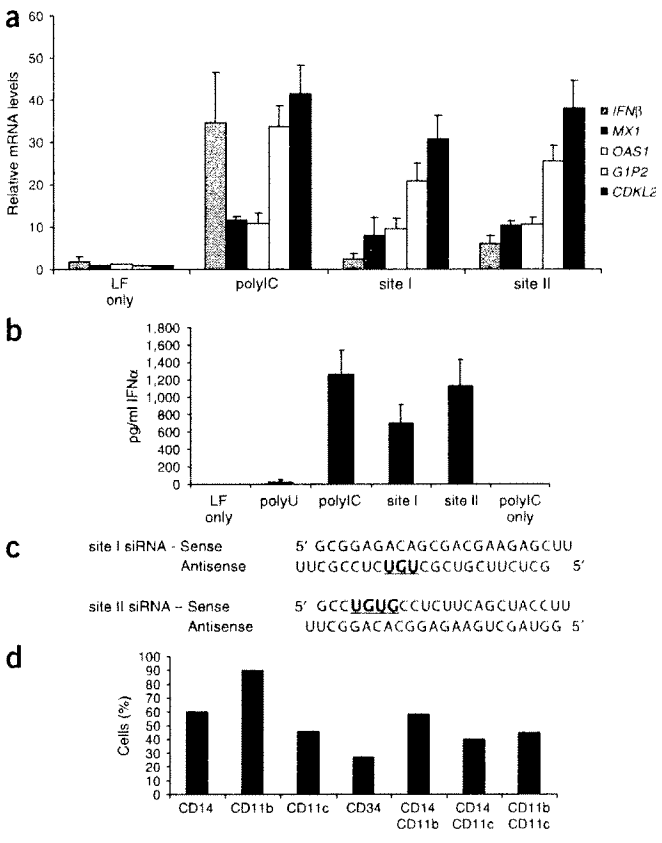

Figure 2 Transfection of synthetic siRNA to site I or site II induces IFNα protein production and IFN-inducible gene expression in CD34$^+$ progenitor-derived monocytes. Cells were treated as in **Figure 1b** except 0.5 μg/ml of RNA was added. (**a**) Real-time PCR was performed for *MX1*, *OAS1*, *G1P2*, *CDKL2* and *IFNβ* mRNA expression. (**b**) IFNα ELISA was performed on supernatants. Error bars represent the standard deviation of triplicate measurements within one experiment. Experiments were performed twice. (**c**) siRNA sequences for site I and site II used in transfections in **a** (described in ref. 22). (**d**) FACS analysis for CD14, CD11b, CD11c and CD34 was performed on CD34$^+$ progenitor-derived monocytes 6 d after addition of M-CSF and GM-CSF for the experiment in **a**.

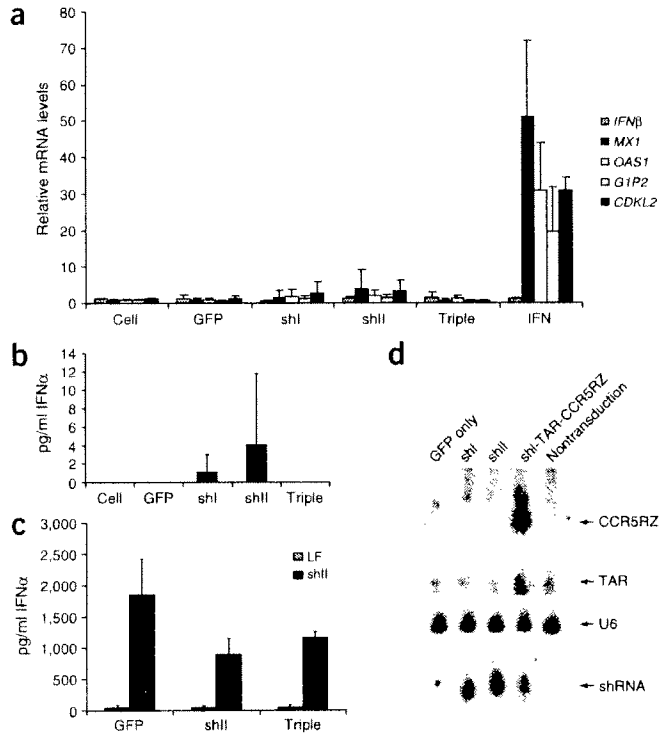

Figure 3 IFN and IFN-inducible gene expression in CD34$^+$ cells transduced with lentiviruses expressing GFP alone, shRNAs to site I or II and the triple construct containing site I shRNA, TAR decoy and chimeric VA1-CCR5 ribozyme. GFP$^+$-transduced CD34$^+$ progenitor cells were either grown in M-CSF and GM-CSF for 8 d after FACs sorting or treated with 1,000 U/ml IFNα in addition to M-CSF and GM-CSF 4 d after sorting. RNA was extracted and (**a**) real-time PCR performed for *IFNα*, *IFNβ*, *MX1*, *OAS1*, *G1P2* and *CDKL2* and *IFNβ* mRNA levels. (**b**) IFNα ELISA was performed on supernatants from cells in **a**. (**c**) CD34$^+$ cells were transduced with either the EGFP vector, or the EGFP vector containing the site II shRNA or the triple construct and differentiated as described above except that after 14 d of differentiation, the cells were transfected with 0.5 μg/ml synthetic shRNA to site II (shII) 5′–GGUAGCUGAAGAGGCACAGGC**UUCG**GCCUGUGCCUC UUCAGCUACCUU-3′ (bold sequence is loop) in complex with Lipofectamine 2000 and the supernatants harvested and examined for IFNα production at 48h after transfection. Blue bars represent control values with lipid vehicle alone, and maroon bars represent stimulation with the lipid-shRNA complex. (**d**) Northern blot analysis and hybridizations were performed using 5 μg of total RNA from **a**. One representative experiment with biological triplicates is shown. Error bars represent the standard deviation. The experiments were performed twice except for **c** which was performed once.

and site II alone resulted in marginally elevated levels of *OAS1*, *MX1*, *G1P2* and *CDKL2* gene expression (**Fig. 3a**).

The lack of *IFNβ* mRNA and IFN-inducible gene expression suggested that type I IFN was not being produced in CD34$^+$ progenitor cells expressing the triple construct. To confirm this, we performed ELISA for IFNα protein on cell supernatants. No IFNα protein was measured in supernatants from control cells or parental HIV7 and triple construct–transduced cells (**Fig. 3b**). However, IFNα was produced at low levels: an average value of 2 pg/ml in cells expressing shRNA to site I alone or 5 pg/ml in cells expressing shRNA to site II alone. These levels are several hundred fold lower than those obtained with the transfected synthetic sequences. To determine whether these transduced cells were capable of mounting a potent IFN response to exogenously added RNA, we transfected a chemically synthesized shRNA to site II into CD34$^+$ progenitor cells transduced with GFP, shII or the triple construct. Strong IFNα production (range of 1,000 pg/ml) was observed (**Fig. 3c**). Northern blot analyses were carried out to verify that these cells were expressing the respective shRNAs. shRNA expression could be detected for both site I and site II shRNAs (**Fig. 3d**). The VA1-CCR5 ribozyme and TAR decoy were also expressed in cells expressing the triple construct. CD34$^+$ progenitor–derived immune cells were protected from HIV-1 challenge when expressing either shRNA to site I or site II or the triple construct (**Supplementary Fig. 3** online). Whereas the single hairpin RNAs reduced HIV-1 p24 antigen by ~50% on day 14, expression of the triple construct completely prevented production of p24 up to 21 d after virus challenge (**Supplementary Fig. 3**), consistent with previous observations[16].

To examine whether shRNA-expressing CD34$^+$ cells differentiate normally in cell culture, we grew CD34$^+$ cells in nonadherent culture and transduced them with lentiviral constructs expressing EGFP with or without an shRNA to site I or site II or the triple construct. Transduction efficiencies with the lentivirus vector ranged from 25% to 30% (**Supplementary Fig. 4a** online). Cells were sorted for EGFP expression 4 d after transduction to ensure that the majority of the cells expressed the shRNAs. The cytokines M-CSF and GM-CSF were added to sorted CD34$^+$ progenitor cells grown in nonadherent liquid culture to induce differentiation to immune cells. Markers for monocyte differentiation were monitored by FACS analysis to determine the phenotypes of the cell populations. CD14 is a primitive monocyte marker and CD11b is a marker of mature monocytes such as macrophages and dendritic cells. Addition of M-CSF and GM-CSF to untransduced CD34$^+$ progenitors increased the percentage of CD14$^+$ and CD11b$^+$ cells, with a subset of these cells being doubly positive for CD14/CD11b, suggesting that a proportion of the CD34$^+$ progenitors were differentiating into more mature monocytes such as macrophages and dendritic cells (**Supplementary Fig. 4b**). The levels of monocytes in control untransduced cells and cells expressing EGFP alone were similar to cells expressing the shRNAs or the combination of three RNAs in the triple construct (**Supplementary Fig. 4c**). These data show that CD34$^+$ cells transduced via a lentiviral vector retain the capacity to differentiate into monocytes in liquid culture and that CD34$^+$ progenitor–derived monocyte differentiation *in vitro* is not compromised by expression of shRNAs.

To determine whether CD34$^+$ progenitor–derived immune cells contained the IFNα-producing cell subset of pDCs[17], we grew and differentiated transduced cells in culture as described above. Approximately 20% of the stably transduced and differentiating CD34$^+$ cell population had differentiated into CD11c-/BDCA4$^+$ pDCs after 14 d of differentiation (**Supplementary Fig. 5** online). In combination, these data show that CD34$^+$ progenitor–derived immune cells retain the capacity to differentiate in culture into pDCs and monocytes even when stably expressing shRNAs.

We next asked whether the cytokine production profiles are altered in CD34$^+$ progenitor–derived immune cells expressing shRNAs. A^2 multiplex immunoassays were used to monitor the cytokine profile in supernatants of cultured CD34$^+$ progenitors and CD34$^+$ progenitor–derived monocytes. The cytokines and chemokines examined were IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, GM-CSF, TNFα, MCP-1, RANTES and IFNγ. Of the 18 cytokines and chemokines measured, only 4 were detected in supernatants of unsorted human CD34$^+$ progenitors or CD34$^+$ progenitor–derived immune cells after 21 d in culture. These were IL-1RA, IL-8, MCP-1 and RANTES (**Fig. 4**). The other 14 chemokines

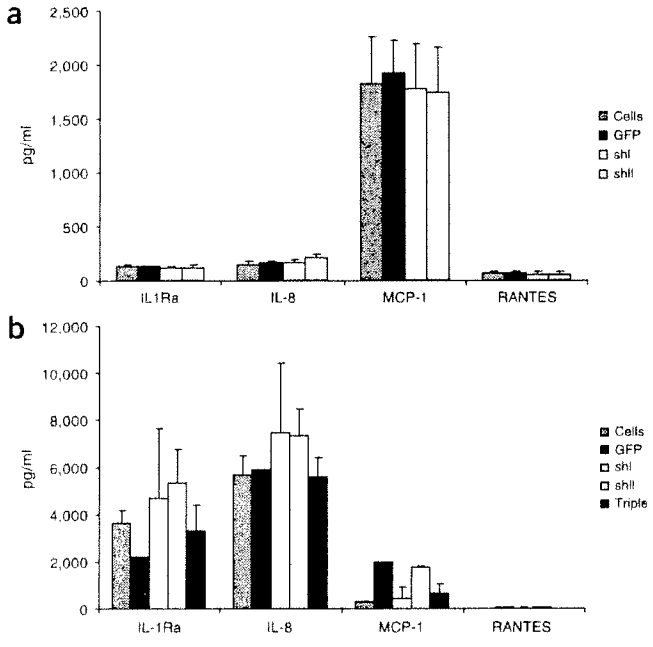

Figure 4 Chemokine and cytokine expression profiles in CD34$^+$ progenitors and CD34$^+$ progenitor–derived monocytes. CD34$^+$ progenitor cells were transduced with lentiviruses expressing GFP alone or with shI or shII. Eight days later, culture supernatants were harvested and stored at –80 °C until A2 multiplex cytokine immunoassays were performed. Seven days after transduction, the cells were either (**a**) maintained in the clinical medium for a further 7 d and then supernatants harvested or (**b**) FACS sorted for GFP expression and the cytokines GM-CSF and M-CSF added after sorting to induce cells to differentiate into immune cells. Eight days later, culture supernatants were harvested and stored at –80 °C until A2 multiplex cytokine immunoassays were performed. Error bars represent the s.d. between two separate experiments.

and cytokines were not detectable in supernatants from control cells or cells expressing shRNA. The levels of IL-1RA, IL-8, MCP-1 and RANTES were similar among control CD34$^+$ progenitor cells and cells expressing the shRNAs or the triple RNA combination (**Fig. 4a**).

To determine whether the cytokine profile and/or levels of cytokine production would change in a sorted population of cells differentiating into various types of immune cells, we performed further experiments using cell supernatants from CD34$^+$ progenitor–derived immune cells expressing shRNAs to site I or site II or the triple RNA combination. Seven days after transduction, these cells were sorted for EGFP expression. A^2 multiplex immunoassays for production of IL-1RA, IL-8, MCP-1 and RANTES were performed with supernatants from these cells 8 d after addition of M-CSF and GM-CSF. After differentiation into immune cells, the profile of cytokine production was the same as with the CD34$^+$ progenitor cells, with detectable expression of IL-1RA, IL-8, MCP-1 and RANTES in all treatments (**Fig. 4b**). The levels of IL-1RA and IL-8 increased in the differentiating cells versus the CD34$^+$ progenitors, but there was no difference in levels between control cells and cells expressing the shRNAs.

To our knowledge, no previous studies have compared the immune response to synthetic siRNAs and expressed shRNAs harboring the same immunostimulatory motifs in cell types that have the complement of TLRs that would promote inflammatory cytokine production and the IFN response. CD34$^+$ progenitor cells from cord blood express the receptors for IFNα and IFNβ, and these receptors are upregulated during CD34$^+$ progenitor cell differentiation[18]. We have confirmed that CD34$^+$ progenitor–derived immune cells can be stimulated by IFNα and have shown that CD34$^+$ progenitor–derived monocytes differentiated *in vitro* can also be stimulated by dsRNA in the form of polyIC, siRNA and shRNA when the RNA is delivered via a lipid vehicle.

As synthetic siRNAs to site I and site II induced a strong IFN-response in CD34$^+$ progenitor–derived immune cells when delivered by lipid vehicle, it was surprising that expression of shRNAs to site I or site II gave minimal, sporadic (in two out of six samples), induction of IFNα and IFN-inducible gene expression. These levels are at least 100-fold lower than IFNα induction by synthetic siRNAs delivered to the cytoplasm via a lipid vehicle. It may be that the CD34$^+$ progenitor cells, when stably expressing shRNAs, do not respond normally to RNA via IFN production. However, we have shown that CD34$^+$ hematopoietic progenitor cells transduced with the GFP, shII and triple RNA constructs do produce substantial IFNα in response to exogenous shRNA to site II (**Fig. 3c**), indicating that the cells retain their ability to respond to immunostimulatory RNAs delivered via lipid vehicle. A possible explanation for the marginal IFN induction by expressed shRNAs versus lipid-delivered synthetic siRNAs/shRNAs is that Pol III–expressed shRNAs, as opposed to lipid-delivered synthetic siRNAs, may not encounter the endosome where nucleic acid–binding TLRs reside. Given the sporadic and extremely low levels of IFNα induction in the cells stably expressing shRNAs I or II, these small differences may not be biologically meaningful.

These results bode well from the perspective of using shRNAs for gene therapy applications. Considering that the immunostimulatory activity of siRNAs is sequence specific, we suggest that siRNAs or shRNAs for clinical applications be examined for immunostimulatory activity in human immune cells as we have done for the expressed shRNAs to site I and site II and the triple construct. In the future, it would be interesting to examine other shRNA sequences to determine whether they can similarly avoid IFN induction when expressed in human hematopoietic stem cells. It should be noted that a clinical trial using the triple construct to transduce autologous CD34$^+$ cells from HIV-1 patients is in the final planning stages. The data presented here provide a level of safety assurance for expression of shRNAs in a hematopoietic stem cell setting.

METHODS

Lentiviral vector production. 293T cells were cultured until they reached 80% confluency in a 100-mm culture dish. Fifteen μg of lentiviral vector with the appropriate insert, 15 μg of pCHGP-2, 5 μg of pCMV-G and 5 μg of pCMV-REV were cotransfected into HEK293T cells using the calcium phosphate precipitation procedure. The culture medium was replaced 6 h after transfection. The culture supernatants were collected at 24 h and 36 h after transfection. The supernatants were pooled together, passed through a 0.2-μm filter, concentrated by ultracentrifugation and stored at –80 °C until use. Vector titers were determined by transduction of HT1080 cells and assayed for EGFP expression using flow cytometry. The vectors were free of replication-competent lentivirus as determined by both RT-PCR and p24 antigen assays (Beckman-Coulter).

Transduction of CD34$^+$ cells. CD34$^+$ hematopoietic stem cells were enriched from umbilical cord blood by anti-CD34 antibody-coupled magnetic beads (Miltenyi Biotech). The purity of CD34$^+$ cells was above 90% as determined by FACS analysis. Cells were frozen in medium containing 10% DMSO at –80 °C until needed. CD34$^+$ hematopoietic stem cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% BIT9500 (Stem Cell Technology), 40 μg/ml human low density lipoproteins (Sigma), 10^{-4} M 2-mercaptoethanol, 100 ng/ml stem cell factor, 100 ng/ml flt3-ligand,

10 ng/ml thrombopoietin (PeproTech). Cells were thawed and washed in IMDM and then prestimulated in the above medium for 4 d. The lentiviral vector stock was adjusted to a multiplicity of infection (MOI) of 40 in 200 μl culture medium and loaded onto RetroNectin-coated 24-well plate (Takara Mirus). After incubation at 32 °C for 4 h, the vector supernatant was removed and the wells were washed with PBS. The prestimulated $CD34^+$ cells were added to the wells at 5×10^4/ml in the growth medium. Cells were washed 24 h later and cultured in complete medium as described above. $CD34^+$ progenitor cells were cultured and transduced with lentiviruses encoding GFP alone or in combination with shRNA to site I or site II or a triple construct encoding shRNA to site I, a CCR5 ribozyme and a Tar decoy *in vitro* and 4 d after transduction the $CD34^+$ progenitors were FACS sorted based on GFP expression. GFP-positive cells were cultured a further 4 d at which point the cytokines GM-CSF and M-CSF were added to induce cells to differentiate into immune cells.

Differentiation of $CD34^+$ progenitors into monocytes. Lentivirus-transduced $CD34^+$ hematopoietic stem cells were either used directly in experiments or sorted for GFP fluorescence by FACS. Nonsorted cells were grown in complete medium until cell numbers were in the range of 0.5 to 1×10^6 cells/ml (~7 d) and then split, one half of the cells grown in complete medium and one half grown in complete medium with addition of 50 ng/ml M-CSF and 20 ng/ml GM-CSF (Peprotech) to induce differentiation to a mixed population of immune cells including pDCs, monocytes and mDCs. Cells were harvested for RNA extraction 8 d after addition of M-CSF and GM-CSF, ~14 d after transduction, for a total of 19 d in culture. Sorted cells were grown in complete medium plus M-CSF and GM-CSF for differentiation into immune cells. The percent monocytes were identified by incubation with fluorescently labeled antibodies to the monocyte markers CD14 and CD11b (Caltag Laboratories) and the percent pDCs and mDCs were determined by incubation with fluorescent antibodies to CD11c (Caltag Laboratories) and BDCA-1 (mDCs) or BDCA-4 (pDCs) (Miltenyi Biotec) according to the manufacturer's instructions followed by FACS analysis. Cells were incubated with anti-CD14 antibody labeled with PE-Cy5.5, anti-CD11b (Mac-1) antibody labeled with APC, anti-CD11c antibody labeled with PE and anti-CD34 antibody labeled with FITC for 20 min, washed and fixed in 3.7% formaldehyde for 30 min followed by FACS analysis for PE-Cy5.5, APC, PE and FITC.

HIV-1 challenge and p24 antigen assay. $CD34^+$ cells were transduced with various lentiviral vectors. EGFP positive ($EGFP^+$) cells were collected by FACS sorting. After recovery from sorting, 5×10^5 cells were exposed overnight to the JR-FL strain of HIV-1 at multiplicity of infection (MOI) of 0.01. The infected cells were washed four times with HBSS and the cells were maintained in the culture medium for $CD34^+$ cells as described above. The culture supernatant was collected on a weekly basis. The p24 antigen analyses were performed using a Coulter HIV-1 p24 Antigen Assay (Beckman Coulter) according to the manufacturer's instructions.

Transfection of $CD34^+$ progenitor–derived monocytes with dsRNA or siRNA. $CD34^+$ progenitor cells from cord blood were differentiated into monocytes for 7 d by addition of M-CSF and GMCSF as described above, at which time 0.5 μg/ml of either synthetic siRNA to site I or site II (Dharmacon) or of synthetic shRNA to site II (IDT) or of dsRNA in the form of polyIC (Sigma) was complexed with Lipofectamine 2000 (Invitrogen) at a ratio of 1 μg RNA to 3 μl of Lipofectamine 2000 according to the manufacturer's instructions, and added to cells. Six hours after addition of complexes to the cells, fresh medium was added. Supernatants were harvested 48 h after transfection and total RNA harvested by resuspending the cell pellet in 1 ml of RNAstat60 (Tel-Test).

Cytokine assays. Cell supernatants were examined for cytokine production either by ELISA or using an A^2 multiplex assay. ELISA for IFNα was performed using the high sensitivity protocol as per the manufacturer's instructions of the human multi-subtype IFNα ELISA Kit (PBL Biomedical). Cells were monitored for production of a panel of 18 cytokine and chemokine assays including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, GM-CSF, TNFα, MCP-1, RANTES and IFNγ using the A^2 Multiplex System according to the manufacturer's instructions (Beckman Coulter). In brief, a mixture of Hybridization Buffer A and B with antibody-oligonucleotide conjugates of an assay panel of 13-plex (IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, GM-CSF, TNFα and IFNγ) or 5-plex (IL-1α, IL-1RA, IL-8, MCP-1 and RANTES) provided with the A^2 Multiplex System was hybridized to the A^2 Universal Plate with 1 h incubation at 24 °C. Cell culture supernatant samples were applied to the wells after washing the plates three times with 1μ wash buffer. After 1 h incubation at 24 °C and three plate washes, a cocktail of biotinylated detection antibodies was applied, followed by an addition of streptavidin-fluor. Assay signals were quantified by the A^2 plate reader and application software. Cytokine concentrations in the samples were calculated by A^2 software from corresponding calibration standards run on the same plate with 4-parameter logistic curve fitting. A^2 cytokine assays have intra-assay CVs of less than 10% and inter-assay CVs of 20% (data not shown). A typical A^2 cytokine assay with cell culture supernatant samples has assay sensitivity of ≤10 pg/ml and an assay range of 20–5,000 pg/ml (data not shown).

RNA expression analysis. Expression of human mRNAs encoding *IFNα*, *IFNβ*, *OAS1*, *MX1*, *G1P2*, *CDKL2* and *HPRT1* were determined using real-time PCR using 2X iQ SyberGreen Mastermix (BIO-RAD) and specific primer sets for these genes at final concentrations of 400 nM. Primers were as follows:

CDKL2 (ref. 19) forward, 5′-GCCTCCTTGGGTTCGTCTATAA-3′ and reverse, 5′-CTCAGGGCCCGCTCATAGTA-3′; *HPRT1* (ref. 20) forward, 5′-TGACACTGGCAAAACAATGCA-3′ and reverse, 5′-GGTCCTTTTCACCAGCAAGCT-3′; *IFNβ* forward, 5′-AGACTTACAGGTTACCTCCGAA-3′ and reverse, 5′-CAGTACATTCGCCATCAGTCA-3′; *OAS1* forward, 5′-CGAGGGAGCATGAAAACACATTT-3′ and reverse, 5′-GCAGAGTTGCTGGTAGTTTATGAC-3′; *MX1* forward, 5′-CTGGTGCTGAAACTGAAGAAAC-3′ and reverse, 5′-ATCTCAATCTCGTAGTCCTGGTA-3′; and *G1P2* (ref. 21) forward, 5′-CATGGGCTGGGACCTGACGGTGAAG-3′ and reverse, 5′-CTGCTGCGGCCCTTGTTATT-3′. RNAStat60 reagent was used to extract total RNA according to the manufacturer's instructions (Tel-Test). Residual DNA was digested using the DNA-free kit per the manufacturer's instructions (Ambion). cDNA was produced using 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and random primers in a 20 μl reaction according to the manufacturer's instructions (Invitrogen). *HPRT1* expression was used for normalization of the real-time PCR data.

Northern hybridization. Equal concentrations of total cellular RNA (5 μg/lane) were loaded in a 6% denaturing polyacrylamide gel, the RNA transferred onto Hybond-N^+ membrane and probed using oligos complementary to the antisense strands of the siRNAs to site I (tat/rev) or site II (rev) or to U6 RNA as described[22]. Probes for the VA1-ribozyme to CCR5 or the nuclear localizing TAR decoy were as described[16].

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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